Directed evolution and axial chirality: optimization of the enantioselectivity of *Pseudomonas aeruginosa* lipase towards the kinetic resolution of a racemic allene†

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Directed evolution of *Pseudomonas aeruginosa* lipase by the use of combinatorial active site saturation test (CAST) criteria provided a highly enantioselective mutant (Leu162Phe) for kinetic resolution of an axially chiral allene, *p*-nitrophenyl 4-cyclohexyl-2-methylbuta-2,3-dienoate (E = 111); the high enantioselectivity of the Leu162Phe mutant was rationalized by π–π stacking.

Biocatalysis has emerged as one of the most important areas of enantioselective organic synthesis.[1,2] The scaling up of biocatalytic processes is rather uncomplicated since many enzymes are readily accessible to a low cost, and furthermore, they can often be easily immobilized and reused.[3] Recently, enzymatic resolution has been successfully combined with *in situ* metal-catalyzed racemization of the substrate, which leads to highly efficient dynamic kinetic resolution (DKR).[4–7] It is of great interest to broaden the substrate tolerance of these enzymes as well as to extend their scope to new types of substrates. In this respect directed evolution is a powerful tool, and substantial progress has recently been made in this area.[8,9]

Allenes are important compounds that have attracted considerable interest during the past decade. They have recently been found to participate in a number of spectacular reactions.[10–12] Allenes possess an axial chirality and we recently reported on a palladium-catalyzed racemization of allenes.[13] The latter racemization if combined with an enzymatic resolution may lead to an efficient DKR process. However, because allenes are rare in nature only limited examples of enzymatic resolution of axially chiral allenes are known in the literature.[14,15] We now report on a mutant from directed evolution of *Pseudomonas aeruginosa* lipase (PAL) that gives very high enantioselectivity ($E > 100$) in the kinetic resolution of an axially chiral allene.

During the development of combinatorial active site saturation test (CAST) method using *Pseudomonas aeruginosa* lipase (PAL) five different saturation mutagenesis libraries (A–E) were selected according to the CAST criteria (see ESI†).[16] These libraries were tested towards a collection of bulky *p*-nitrophenyl esters with the objective to expand the substrate scope, and in parallel to enhance the enantioselectivity in the kinetic resolution of *p*-nitrophenyl 2-methyldecanoate. The wild-type enzyme gave a very low enantioselectivity (E = 1.2 (*S*)) with the latter substrate. Although several of the libraries presented some interesting results, the mutants showing a real improvement were all from the same library (library D).

We therefore decided to try the CAST strategy for the kinetic resolution of an allene structure and to point out that it is possible to apply directed evolution by means of CASTing to increase the enantioselectivity of an enzyme versus axial chirality.

The *p*-nitrophenyl ester **1** was chosen as substrate, due to its similarity with the non-allenic substrates, such as **2** (Fig. 1), that previously led to positive results with the designed libraries.[16a]

The wild-type lipase of *Pseudomonas aeruginosa* gave a moderate E value (E = 8.5) in the enantioselective hydrolysis of **1** showing preference for the (+)-enantiomer (*cf.* Table 1, entry 6). The corresponding kinetic resolution of substrate **2** with the wild-type enzyme is almost stereorandom (E = 1.2).

Due to the limitations of the evaluation of the enantioselectivity by gas chromatography (~1 h/sample), we were forced to dramatically reduce the size of the library to be screened. From a previous screening of 15000 variants,[16] a total of 600 mutants had been selected that showed different behaviour compared to the wild-type in terms of enantioselectivity towards the kinetic resolution of a structurally related ester **2** or in terms of substrate scope towards other *p*-nitrophenyl esters.[16] These 600 mutants were screened towards enantioselective hydrolysis of **1**.

From the screening, mutants with a higher enantioselectivity compared to the wild-type in kinetic resolution of **1** were found as well as mutants that showed the reverse enantiopreference (selectivity for the (−)-enantiomer) (Table 1). As for **2**, the best results for the allenic substrate **1** were obtained with library D variants (mutants of residues Leu159 and Leu162). The best mutant obtained harbors a single mutation Leu162Phe (L162F) that enhances the enantioselectivity of the enzyme from E = 8.5 for the wild-type to E = 111 (showing the same enantiopreference, giving the (+)-isomer) (Table 1, entry 1). This mutant was superior to all lipases tested for the kinetic resolution of **1** (ESI†) and the E is by far the highest known for axially chiral allenes. Due to the

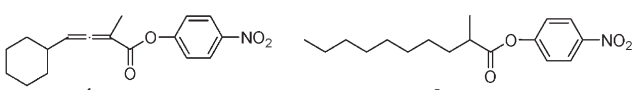

Fig. 1

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Table 1 Kinetic resolution of the *p*-nitrophenyl allene ester **1** using different mutants from *Pseudomonas aeruginosa* lipase

Entry	Variant	Library/ source	ee[a]	Conv. (%)	*E*
1	Leu162Phe	D	96 (+)	44	111
2	Leu162Val	D	91 (+)	40	39
3	Leu162Ile	D	90 (+)	39	33
4	Leu162Ala	D	82 (+)	29	13
5	Leu162Thr	D	77 (+)	23	10
6	*P. aeruginosa* lipase	WT	74 (+)	25	8.5
7	Leu162Asn	D	75 (+)	20	8.4
8	Leu159Trp/Leu162Thr	D	68 (+)	17	6
9	Met16Ala, Leu17Phe	A	59 (+)	23	4.2
10	Val232Ile	E	56 (+)	31	4.5
11	15B10[b]		55 (+)	17	3.9
12	Leu231Ile/Val232Cys	E	42 (+)	28	2.8
13	Leu162Gly	D	20 (−)	18	1.6
14	Leu162Gly/Leu159Tyr	D	16 (−)	24	1.5
15	Leu162Gly/Leu159Val	D	15 (−)	22	1.4
16	1H8[c]		26 (−)	15	1.8

[a] Enantiomeric excess of the acid (4-cyclohexyl-2-methylbuta-2,3-dienoic acid). The first eluting enantiomer of the acid has a positive rotation (+). [b] 15B10 represents the following mutations: V232I, M16L, A34T, P86L, D113G, S237T, T150A, S147N, V94A, T87S, L208H. [c] 1H8 represents the following mutations: D20N, S53P, S155M, L162G, T180I, T234S

high degree of enantioselectivity displayed by the best hit in the initial library D of mutants, it was not necessary to engage in an evolutionary process by performing iterative CASTing.

The dramatic increase of the *E* value from 8.5 to an *E* value of over 100 on changing leucine to phenylalanine at position 162 (Leu162Phe), as well as the considerable effects found with other amino acid exchanges (Leu162Val, $E = 39$ and Leu162Ile, $E = 33$) (Table 1, entries 2 and 3), is especially remarkable as position Leu162 was previously found to be critical for the enantioselectivity in the kinetic resolution of **2**. In the latter case the single mutation responsible for the most dramatic effect on the enantioselectivity was found by changing leucine for glycine at this position (Leu162Gly), which improved the *E* value from 1.2 (*S*-selective) of the wild-type to an *E* value of 32 (*S*-selective) in the hydrolysis of **2**.[17] Also for the kinetic resolution of **1**, variant Leu162Gly results in a significant enantioselectivity change, inverting the enantioselectivity to give the (−)-isomer with $E = 1.6$ (Table 1, entry 13).

In general, a given mutant provides similar consequences in terms of enantioselectivity towards substrates **1** and **2**. Thus, mutant 1H8 (D20N, S53P, S155M, L162G, T180I, T234S) and

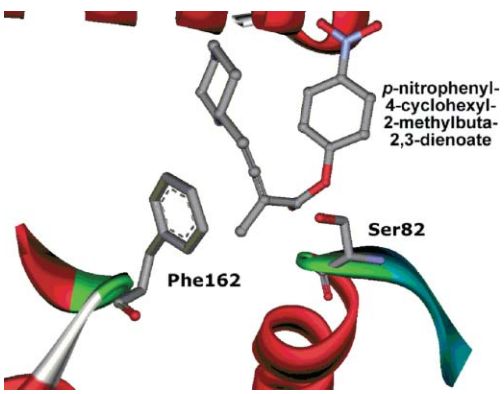

Fig. 2 Substrate **1** docked. Catalytic Ser82 and Leu162Phe.

Table 2 Energies of the presumed tetrahedral intermediates for the hydrolysis of **1** using the wild-type lipase of *Pseudomonas aeruginosa* lipase and the mutants Leu162Gly and Leu162Phe

	Energy/kcal mol^{-1}		
Variant	*R*-tetrahedral intermediate	*S*-tetrahedral intermediate	Δ ($R - S$)
WT	−18.1	−16.1	+2.0
Leu162Gly	−23.2	−28.8	−5.4
Leu162Phe	−20.7	−17.4	+3.3

mutant 15B10 (V232I, M16L, A34T, P86L, D113G, S237T, T150A, S147N, V94A, T87S, L208H) were previously found to give the best (*R*)- and (*S*)-selectivity, respectively in the kinetic resolution of **2** after several rounds of directed evolution.[18] Also, for the kinetic resolution of substrate **1** these two mutants show opposite enantioselectivity, albeit with low *E* values (Table 1, entries 11 and 16).

Interestingly, mutant Leu162Phe shows a much larger enhancement of the enantioselectivity in the kinetic resolution of **1** ($E_{L162F} > 100$, $E_{wild\text{-}type} = 8.5$) compared to the kinetic resolution of **2** ($E_{L162F} = 5$, $E_{wild\text{-}type} = 1.2$) and this observation may be explained by the π–π stacking effect between the phenyl side chain of phenylalanine 162 and the π-electrons of the allene bond (Fig. 2). This interaction may affect the orientation of the methyl group in the 2-position of **1**, favoring the recognition of one of the enantiomers. In the case of **2**, this type of interaction is not possible. The π–π stacking effect is very important for the stabilization of the protein structure as previously described[19] and may also be responsible for the major changes in the interaction with the substrate and in the conformation of the substrate binding site.[16b,20]

The energies of the presumed (*R*) and (*S*) tetrahedral intermediates in the hydrolysis of the *p*-nitrophenyl allene ester **1** catalyzed by the wild type enzyme and variants Leu162Gly and Leu162Phe were calculated with MOLOC using the same protocol as previously published.[21] In that study it was shown[21] that the tetrahedral intermediate is representative for the rate-limiting catalytic step in ester hydrolysis. Thus, we can relate the difference in stabilization energy between the two enantiomers of the modeled tetrahedral intermediates directly with the observed enantioselectivity. The results confirmed the inversion in enantioselectivity in the case of Leu162Gly and the enhanced enantioselectivity with Leu162Phe (Table 2).

In conclusion, directed evolution by means of CASTing and iterative–CASTing has proven to be a fast and reliable way to increase the enantioselectivity of an enzyme.[22] In this work a highly enantioselective mutant for kinetic resolution of an axially chiral allene was found by screening a reduced library of 600 mutants obtained by CASTing.

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